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**For:** CLONING AND EXPRESSION OF A  
NEW MCP RECEPTOR IN GLIAL CELLS

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Cloning and expression of a new MCP receptor in glial cells

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Title: Cloning and expression of a new MCP receptor in glial cells

5           The invention relates to the fields of inflammation and immunology, and more specifically to the field of chemokines and receptors therefor, and their role in neurodegenerative or neuroinflammatory disease.

10           Chemokines are small chemotactic cytokines of approximately 10kDa, which orchestrate the inflammatory response by attracting leukocytes to sites of inflammation and by controlling the homing of dendritic cells, T cells and B cells (for review see: 1, 2, 3). Chemokines and their receptors, all of which are G-protein coupled, are subdivided into four families: CXC-, CC-, C- and CX3C-chemokines (3). Chemokine signaling is highly promiscuous, most chemokines activate more than one chemokine receptor and vice versa (4, 5). In humans more than 25 CC chemokines and 10 CC chemokine  
15 receptors (CCR) have been cloned (3). Furthermore, it is likely that some of the currently known orphan chemokine receptors will make chemokine signaling even more complex (4).

20           Chemokines and their receptors are not only found in the peripheral immune system. It has become clear recently that chemokines are also expressed in brain during development and brain pathology (for review see: 6, 7, 8, 9). One of the first described and most prominent chemokines in brain is monocyte chemoattractant protein-1 (MCP), which is found in brain tissue after ischemia (10, 11), Alzheimer's disease (12), and Multiple sclerosis (13, 14 15). Within the damaged brain MCP-1 is produced by both astrocytes and microglia (10) and mediates, presumably, the infiltration by  
25 monocytes/macrophages and lymphocytes (16, 17). Both astrocytes and microglia are not only capable to produce chemokines. They also are involved in chemokine signaling since it is known that glial cells itself express functional chemokine receptors (18, 19, 20). In cultured microglia and astrocytes MCP-1 induces transient increases in intracellular  $Ca^{2+}$  and/or chemotaxis (20, 21, 22, 23, 24). Although cultured glial cells  
30 (astrocytes and microglia) respond to MCP-1 stimulation, possible mRNA expression of the corresponding chemokine receptor for MCP-1 (CCR 2) (25, 26) is controversial in glial cells (18, 24, 9), and so far CCR2 mRNA expression has not been found in astrocytes.

The invention provides the insight that, possibly instead of activating the CCR-2 receptor, in brain cells such as glial cells MCP-1 activates at least one other CC chemokine receptor, a receptor earlier known as orphan receptor L-CCR in the mouse or CRAM-B in humans, which we from now on will address as CCR12, see also figure 8.

5 MCP-1 induced chemokine receptor activation is therefore now shown to be involved in brain pathological events such as neurodegenerative and/or neuroinflammatory disease. A new chemokine ligand-receptor pair is thus found that contributes to an endogenous inflammatory cascade in the central nervous system which is related to above identified pathological conditions.

10 With that insight the invention for example provides a method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising testing said compound for its capacity to modulate MCP-1 binding with an orphan receptor commonly known as L-CCR in the mouse or CRAM-B in humans, in particular for the treatment of brain disease after ischemia, Alzheimer's  
15 disease or multiple sclerosis. The invention provides for example the characterization and the observation of mRNA expression of a novel MCP chemokine receptor CCR12 in glial cells. Evidence is here presented that astrocytes and microglia express mRNA encoding said new chemokine receptor provided here. Cloning and expressing of this new chemokine receptor revealed that MCP-1 is a chemokine ligand for this new  
20 receptor. According to the chemokine receptor nomenclature rules we suggest to designate this receptor CCR12. Since CCR12 mRNA was strongly induced by treatment with LPS both *in vitro* and *in vivo* the further insight is provided here that this receptor plays an important role in brain immunology or brain inflammatory disease.

We thus present evidence for a new MCP-1 chemokine receptor, previously, when  
25 its affinity for MCP-1 was not known, described as the orphan receptor L-CCR (27). L-CCR mRNA is expressed in mouse astrocytes and microglia and regulated by LPS both *in vitro* and *in vivo*. Since it is now found that MCP-1 is a chemokine ligand for L-CCR we designate L-CCR as CCR 12, a new chemokine receptor responsible for the well known effects of MCP-1 on glial cells. The mRNA expression of the CC chemokine  
30 receptors CCR1-5 in cultured glial cells has been, at least partially, investigated by several groups and most studies have been performed with rat and human glial cells (Table 4). Whereas, cultured astrocytes from rat and human did not show any CCR mRNA expression, expression of CCR1 mRNA was found in mouse astrocytes (19). In rat and human microglia mRNA expression of CCR1 (19, 20, 32) and CCR5 (18, 33, 20,  
35 34, 32) has been reported. There are conflicting reports on the expression of CCR2 and



CCR3 mRNA in cultured microglia cells. Low CCR2 mRNA expression was found in cultured microglia by Boddeke et al. (20) and McManus et al., (32), whereas no CCR2 mRNA in cultured microglia was found by others (18, 24). CCR3 mRNA expression in cultured microglia was found by He et al. (33) and McManus et al., (32) but not by Jiang et al. (18) and Boddeke et al., (20). The three reports investigating possible expression of CCR4 mRNA in glial cells failed to detect CCR4 mRNA expression (19, 20, 32). The reasons for the opposite findings concerning expression of CCR2 and 3 mRNA are currently not clear, but might be due to species variations, different culture conditions and/or different detection techniques used (Table 4).

Since very little data are available from the literature concerning CCR mRNA expression in mouse glial cells, we investigated possible mRNA expression of CCR1 to 8 and D6 in cultured mouse microglia and astrocytes using RT-PCR. The mouse chemokine receptor D6 was included in our study since it has been described as mouse CCR9 with MCP-1 binding properties (35). However since MCP-1 signaling for D6 could not be reproduced (36) this receptor was not designated as CCR9 by the nomenclature committee and currently keeps its orphan name D6 (3). All primers used in RT-PCR experiments were positively verified using genomic mouse DNA as a template and subsequent cloning and sequencing of the PCR product. We observed mRNA expression for CCR1,3,5 and CCR1,5 in cultured microglia and astrocytes, respectively, which is in good accordance with the recent literature. No other CCR mRNA's were found.

The results clearly show, that although both microglia and astrocytes respond to MCP-1 (24; own results) cultured mouse glial cells did not express CCR2 mRNA, the receptor responding to MCP-1 or D6 mRNA, a receptor which has binding properties to MCP-1. It is thus likely that cultured mouse glial cells express another receptor for MCP-1, as was already suggested by Heesen et al., (24). RT-PCR and *in situ* hybridisation showed that both cultured astrocytes and microglia express CCR12 mRNA. The CCR12 mRNA expression in both cell types was strongly increased by stimulation with LPS. Similar results were also observed *in vivo*, where CCR12 mRNA expression in cortical glial cells was strongly induced by an intraperitoneal injection of LPS.

These results therefore clearly indicate that mouse glial cells (*in vitro* and *in vivo*) express an additional LPS regulated chemokine receptor which has not been described in glial cells before. LPS stimulated RAW 264.7 cells and CCR12 transfected HEK cells, which both express CCR12, responded in a concentration-dependent manner to MCP-1 in a chemotaxis assay indicating that MCP-1 is a CC chemokine ligand for

CCR12. Except from MCP-1 several other chemokines (RANTES, MIP-1a, MIP-1b, MIP-3a, MCP-2, MCP-3, fractalkine, IP-10 and SLC) are known to be expressed in brain (6-9; own observations). Among these only RANTES, MCP-2 and MCP-3 were agonists for CCR12. The members of the MCP family MCP-1, 2 and 3 are known to activate CCR2, but RANTES is not a chemokine ligand for CCR2, which indicates that the pharmacological profile we found for CCR12 is new and unrelated to the "ligand" profiles of other receptors (3). Taken together our data provide the insight that the effects of MCP-1 on cultured mouse glial cells described in the literature and described here are mediated via CCR12.

Due to multiple cloning and nomination, the nomenclature of chemokine receptors has been confusing in the past (3). In order to exclude that CCR12 encodes an already known CCR paired sequence alignment was performed. Paired sequence alignment of CCR12 with all other known mouse CC chemokine receptors (CCR1-10 and D6) revealed a percentage ID between 48% and 56% on the nucleotide level, indicating that the glial CCR12 encodes a new chemokine receptor (Table 2). This assumption is corroborated by our pharmacological findings that next to members of the MCP family also RANTES was able to activate CCR12. Cloning of the human analogue and its expression in HEK cells revealed that MCP-1 is a chemokine ligand for the human CCR12.

Investigating the binding of biotinylated MCP-1 and MIP-1 $\alpha$  in cultured human astrocytes, it was shown that both chemokines bind to pharmacological different receptors since binding of MCP-1 was not competitively inhibited by MIP-1 $\alpha$  and vice versa (37). CCR1-5 mRNA expression in human astrocytes, however, has not been found in a recent study (32). Due to the data we provide here expression of CCR12 now offers a explanation of the pharmacological data on MCP-1 binding presented by Andjelkovic et al. (37) in human astrocytes.

Among other chemokines such as MIP-1 $\alpha$ , RANTES, IP-10, MCP-2, MCP-3 and C10, MCP-1 is one of the most prominent chemokines in brain. MCP-1 is induced during most types of brain injuries including as Multiple sclerosis, Alzheimer's Disease and Stroke (10-15). Within in the brain predominately glial cells (astrocytes and microglia) are the cellular source of MCP-1 (13, 38, 10, 15). MCP-1 derived from glial cell is considered to be a factor controlling and/or initiating the infiltration of the damaged brain by leukocytes (17). This assumption is corroborated by a variety of findings obtained from cultured glial cells. It was found that cultured astrocytes and microglia

synthesize MCP-1 upon a variety of different stimuli including LPS, IL-1 $\beta$ , INF- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$  and  $\beta$ -amyloid (39-41). Moreover, MCP-1 derived from cultured astrocytes directs the migration of leukocytes across a blood-brain barrier model (16) and the secretion of metalloproteinases by cultured microglia was strongly induced by stimulation with MCP-1 (21).

Brain cells (neurons and glial cells) express various receptors for chemokines such as CCR1 and 5; CXCR2 and 4 and CX3CR (for review see: 9). The expression of chemokine receptors in all intrinsic brain cells provide the insight that chemokines contribute to an endogenous inflammatory cascade in the central nervous system which is related to pathological conditions (42). Effects of chemokines on brain cells such as neuroprotection in hippocampal neurons (43), inhibition of microglial activation (44) and secretion of metalloproteinases by microglia (21) are in line with that insight. Expression of glial CCR12 mRNA *in vitro* and *in vivo* was strongly upregulated by LPS treatment, which shows that CCR12 plays an important role in the chemokine/cytokine signaling cascade during brain inflammation.

The invention provides among others a method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising testing said compound for its capacity to modulate or mimic MCP-1 binding with a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CCR12 and herein also named CCR-12. Such a method is for example useful for finding pharmaceutical compositions for example for the treatment of ischemia, Alzheimer's disease or multiple sclerosis. In particular, such a method is useful when compounds are tested for their capacity to modulate or mimic MCP-1 binding which further comprises down-regulation of said receptor, e.g. for their antagonistic characteristics. Testing can be done *in vitro* or *in vivo*, and the invention provides cells or animals provided or transfected with a recombinant nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CCR12, or functional equivalent thereof, for use in such a method according to the invention.

In a preferred embodiment, testing is provided under circumstances wherein mRNA expression of said receptor is up-regulated, such as to or example mimic inflammatory conditions as can be obtained after treatment with lipopolysaccharide (LPS). In the detailed description a method according to the invention is provided wherein said capacity to modulate or mimic MCP-1 binding is measured by determining

chemotaxis and/or calcium signalling, however, other ways of determining receptor-ligand binding are well known in the art, and can be used as well.

It is for example provided to use said chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof in a method according to the invention  
 5 separate from cells, i.e. in a cell-free system whereby the receptor (or ligand) may be bound to a solid phase and the capacity of the candidate compound is determined by competitive assay or affinity testing.

Preferred is a use according to the invention wherein said receptor or functional  
 10 equivalent thereof is expressed in a cultured cell (*in vitro*) to better mimic pathological conditions, especially when said cultured cell comprises a cell transfected with a nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof, as is for example shown in detail in the detailed description for a HEK cell comprising a recombinant nucleic acid  
 15 encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof. Alternatively, a transgenic mouse (e.g. a knock-in or a knock-out mouse for the nucleic acid in question) is provided for such use, especially when testing in a live animal or tissue therefrom is required.

The invention thus provides a method for obtaining or identifying an agonist or  
 20 antagonist of neurodegenerative of neuroinflammatory disease comprising testing a candidate agonist or antagonist compound in a method according to any one of claims 1 to 7 and determining said compound's capacity to modulate or mimic MCP-1 binding to said receptor in said method, and provides such agonists and antagonists for use for the preparation of a pharmaceutical composition, in particular for the treatment of  
 25 neurodegenerative of neuroinflammatory disease such as Alzheimers disease, stroke, Parkinson's disease, ALS, multiple sclerosis, but use with other (chronic) inflammatory disease, such as atherosclerosis, arthritis, asthma or rheuma is also foreseen, in particular to stop the progression of above mentioned (neuro)degenerative or (neuro)inflammatory diseases.

In summary we show here evidence for the expression of a new LPS regulated chemokine receptor in glial cells *in vitro* and *in vivo*. Also, members of the MCP family and RANTES have been identified as chemokine ligands for this receptor, and we provide the insight that the known effects of MCP-1 on mouse glial cells are mediated via CCR12-induced signaling. What is more, since expression of CCR12 mRNA was  
 35 highly dependent on LPS treatment we provide the insight that CCR12 participates in

the chemokine signaling cascade during brain inflammation. The invention is further explained in the detailed description without limiting it thereto.

Detailed description

Material and methods

## 5 Chemicals

Isoflurane (Forene™) from Abbott (Baar, Switzerland). Dulbeccos modified Eagle Medium from GibcoBRL Life Technologies (Breda, Netherlands); TA vectors pCR2.1 and pCRII from Invitrogen (Leek, Netherlands); digoxigenin-conjugated UTP and alkaline phosphatase conjugated sheep-anti-digoxigenin from Boehringer Mannheim (Mannheim, Germany); recombinant mouse chemokines from Pepro Tech EC Ltd (London, United Kingdom); antibodies for GFAP, ED-1 and MAC-1 from Chemicon (Temecula, USA); Fura-2 AM and all other chemicals from Sigma-Aldrich (Bornhem, Belgium).

15

## Injection of LPS

For treatment with endotoxin, 5 week old CD-1 mice were injected intraperitoneally with LPS (50ug/25g weight) dissolved in sterile saline solution. Control animals received injections with 0.9% NaCl alone. At different time points after the injection, animals were decapitated under isoflurane anaesthesia (5 animals per timepoint, 3 for RNA preparation and 2 for in-situ hybridisation) and brains were removed. Brains were lysed in GTC solution for RNA preparation and fixed with Zamboni's fixative by perfusion fixation for in-situ hybridisation experiments.

25

## Cell cultures

### *RAW 264.7 and HEK 293 cells*

Both RAW 264.7 and HEK 293 cells were maintained in DMEM containing 10% fetal calf serum with 0,01% penicillin and 0,01% streptomycin in a humidified atmosphere (5% CO<sub>2</sub>) at 37° C.

### *Mixed astrocyte cell cultures and cultured microglia*

Mixed astrocyte cell cultures were established as described previously (28). In brief, mouse cortex was dissected from newborn mouse pups (< 1d). Brain tissue was gently dissociated by trituration in phosphate buffered saline and filtered through a cell strainer (70µm Ø, Falcon) in DMEM. After two washing steps (200 x g for 10 min), cells

were seeded in culture dishes (Nunc, 10cm Ø) ( $8 \times 10^6$  cells/dish). Cultures were maintained 6 weeks in DMEM containing 10% fetal calf serum with 0,01% penicillin and 0,01% streptomycin in a humidified atmosphere (5% CO<sub>2</sub>) at 37° C. Culture medium was changed the second day after preparation and every six days thereafter.

- 5 Microglia cultures were established as described previously (29). In brief, floating microglia were harvested from confluent mixed glial cultures and plated on new culture dishes. Microglia cultures were pure (> 95%) as tested by cell specific markers (ED-1 and Mac-1). For calcium measurements cells were seeded on glass coverslips. For chemotaxis assays cultured microglia were left in suspension.

10

#### Reverse transcripts polymerase chain reaction (RT-PCR)

Cells and brain material were lysed in guanidinium isothiocyanate/mercaptoethanol (GTC) buffer and total RNA was extracted with slight modifications according to

- 15 Chomczynski and Sacchi (30).

a) Reverse transcription: 1µg of total RNA was transcribed into cDNA as described (28). Potential contaminations by genomic DNA were checked for by running the reactions (35 cycles) without reverse transcriptase and using GAPDH primers in subsequent PCR amplifications. Only RNA samples which showed no bands after that procedure were

20

b) Polymerase chain reaction: 2µl of the RT-reaction were used in subsequent PCR amplification as described (28). See table 1 for primer sequences, cycle numbers and annealing temperature. Identification of all PCR products were checked by cloning into PCR2.1 (Invitrogen) and subsequent sequencing.

25

#### Cloning and expression of CCR12 in HEK cells

Primers to amplify the full length sequence for mouse CCR12 have been chosen according the sequence for L-CCR (Accession number: AB009384). The full length mouse CCR12 coding sequence was amplified from cDNA derived from LPS stimulated

30 microglia with the following primers: forward, 5'-TATCAAGCAACCTGCCTCAA; backward 5'-TGGCATAAAACAATGTGAAGAGA.

Sequence similarity searches using the mouse CCR12 sequence and human databases gave high homology of mouse CCR12 with the human orphan chemokine receptor CRAM-B (Accession number: AF015525). The following primers were designed to get the

full length sequence for the human CCR12. Forward, 5'-CCCAGTGGGCAGTCTGAA; backward, 5'-CTTGCATTTGGTGGATGCTA.

The resulting PCR products were cloned in PCR2.1 (Invitrogen) for sequencing and subcloned into the *Bam*H I-*Not* I sites of pcDNA 3.1 (Invitrogen) for transfection. 1 µg of the plasmid was transfected with 6 µl Fugene (Roche Molecular Biochemicals) in HEK 293 cells according to the manufacturer's instructions. Stably transfected cells were selected with G418 500 µg/ml for approx. 2 weeks and the resulting cell clones were checked by RT-PCR for CCR12 mRNA expression.

#### Alignment of mouse CCR12 with other CCR's

Paired alignment of the mouse CCR12 with other CCR's was performed using the alignment tool ClustalW at European Bioinformatics Institute (EBI), homepage <http://www.ebi.ac.uk>.

#### Determination of intracellular calcium

For calcium measurements, cells were cultured on poly-L-lysine coated glass coverslips. In order to load the cells with Fura-2 AM the cells were incubated for 30 min at 37°C in loading buffer containing: (in mM) NaCl 120, HEPES 5, KCL 6, CaCl<sub>2</sub> 2, MgCl 1, glucose 5, NaHCO<sub>3</sub> 22, Fura-2 AM 0.005; pH 7.4. The coverslips were fixed in a perfusion chamber (37° C) and mounted on an inverted microscope. Fluorimetric measurements were done using a sensicam CCD camera supported by Axolab<sup>R</sup> 2.1 imaging software. Digital images of the cells were obtained at an emission wavelength of 510 nm using paired exposures to 340 and 380 nm excitation wavelength sampled at a frequency of 1 Hz. Fluorescence values representing spatial averages from a defined pixel area were recorded on-line. Increases in intracellular calcium concentrations were expressed as the 340/380 ratio of the emission wavelengths. Compounds were administered using a pipette positioned at a distance of 100-300 µm from the cells.

#### Chemotaxis assay

Cell migration in response to chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe). Chemokine stock solutions were prepared in PBS and further diluted in medium for use in the assay. Culture medium without chemokines served as a control in the assay. 27 µl of the chemoattractant solution or control medium were added to the lower wells, lower and upper well were separated by a polyvinylpyrrolidone-free polycarbonate filter (8 µm pore size) and 50000 cells per 50 µl



were used in the assay. Determinations were done in hexaplicate. The chamber was incubated at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere for 120 min. At the end of incubation the filter was washed, fixed in methanol and stained with toluidine blue. Migrated cells were counted with a scored eyepiece (3 fields (1mm<sup>2</sup>) per well) and  
 5 migrated cells per chamber were calculated. The data are presented as mean values  $\pm$  S.D. and were analysed by Students *t*-test. P values  $\leq$  0.01 were considered significant.

#### Immunohistochemistry and *in-situ* hybridisation

10 Immunohistochemistry and *in-situ* hybridisation was carried out as described (31). In brief, prior to immunohistochemical processing and between the incubation steps, the sections were washed in 0.9% saline dissolved in 0.05 M Tris, pH 7.4 (TBS). All antisera were diluted in TBS containing 0.3% Triton X-100, 1% bovine serum albumin (BSA) and heparin (5mg/ml). Sections were preincubated in 5% BSA in TBS for 30 min and  
 15 incubated overnight with GFAP and ED-1. Antibody-antigen reactions were detected using the biotin-streptavidin method and the complex was visualised with diaminobenzidine (DAB)/H<sub>2</sub>O<sub>2</sub>. In case of fluorescence detection FITC conjugated streptavidin was used to visualise the antibody-antigen complex. For *in-situ* hybridisation CCR12 PCR product was cloned into the dual promoter PCR II vector and  
 20 linearised. CCR12 sense and antisense probes were synthesised by run off transcription and the use of digoxigenin-conjugated UTP according to the manufactures protocol (Boehringer Mannheim). Slides were rinsed in PBS and digested with 10  $\mu$ g/ml proteinase K for 0.5 h at 37 °C. Subsequently, sections were rinsed in 2x SSC (1 x SSC: 150 mM NaCl, 15 mM Na citrate), dehydrated in an ethanol series and dried.  
 25 Sections were hybridised overnight at 60 °C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, 1 x Denhardt's solution and 10% dextran sulfate. Final probe concentrations in hybridisation buffer were 1-5 ng/ $\mu$ l. After hybridisation, the sections were treated with 10  $\mu$ g/ml of ribonuclease A for 0.5 h at 37 °C and washed  
 30 in 0.1x SSC at 65 °C.

The immunological detection of the digoxigenin labeled RNA-RNA complex was preceded by a 0.5 h pre-incubation at room temperature in 0.1 M Tris, 0.15 M NaCl, pH 7.5 (buffer 1), containing 5% BSA. Slides were incubated for 2h at room temperature with the alkaline phosphatase conjugated sheep-anti-digoxigenin, diluted 1:500 in buffer

1, containing 2% BSA. After thorough rinsing in buffer 1 and a 10 min pre-incubation in an alkaline buffer solution (ABS: 0.1 M Tris, 0.1 M NaCl, 0.05 M  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH 9.5), the alkaline phosphatase was revealed with a freshly prepared solution of 0.34 mg/ml nitroblue tetrazoleum and 0.17 mg/ml 5-bromo-4-chloro-3 indolyl phosphate in ABS.

- 5 Endogenous non-intestinal phosphatase activity was inhibited by the addition of levamisole (0.24 mg/ml) to the staining solution. The color development was done overnight and terminated by placing the slides in a buffer solution, consisting of 0.01 M Tris, 1 mM EDTA, pH 8.5. The dark purple precipitate indicating the presence of hybridised mRNA was revealed with bright-field microscopy. Control experiments  
10 included hybridisation with digoxigenin-labeled sense probes and hybridisation after treatment of the sections with RNase.

## Results

### 15 *Expression of CCR mRNA's in RAW cells*

- The expression of an orphan LPS inducible chemokine receptor (L-CCR) in the mouse macrophage cell line RAW 264.7 was previously described by Shimada et al. (27). Later, we came to designate L-CCR as CCR12, and we use this term hereinafter for the sake of convenience. In order to investigate possible mRNA expression of this receptor in other  
20 cell types, we designed primers for RT-PCR experiments and validated the primers using cDNA derived from RAW 264.7 cells. Results similar to those described by Shimada et al., (1998) were found; mRNA for CCR12 was strongly upregulated in RAW cells by stimulation with LPS (100ng/2h) (Fig. 1). Using RT-PCR analysis (35 cycles) no other mRNA encoding mouse CCR's (CCR1-8 and D6) was detected in cDNA derived  
25 form control or LPS stimulated RAW 264.7 cells. This indicates that CCR12 is the only  $\beta$ -chemokine receptor in these cells. Genomic mouse DNA served as a positive control for the primers (CCR1-8 and D6) used.

### *Expression of CCR mRNA in cultured mouse astrocytes and microglia*

- 30 In cultured mouse microglia mRNA for CCR1, 3 and 5 was detected (Fig 2A). No mRNA for CCR's 2,4,6,7,8 and D6 was found in these cells (35 cycles RT-PCR) (data not shown). Untreated microglia did show basal expression levels for CCR12 mRNA and this expression was upregulated by a 2h stimulation with 100ng/ml LPS (Fig. 2B). Similar but less pronounced effects were found after 2h stimulation with 10 and 1 ng/ml

LPS (data not shown). LPS induction of CCR12 mRNA in cultured microglia peaked at 2h and declined to baseline expression after 8h (data not shown).

Using RT-PCR, mRNA expression for CCR1 and 5 was detected in cultured astrocytes (Fig 2C). No other CCR mRNA (2,3,4,6,7,8 and D6) was found in these cells (35 cycles

5 RT-PCR) (data not shown). Similar to microglia, untreated cultured astrocytes showed basal mRNA expression for CCR12, which also was upregulated after a 2h stimulation with LPS (100ng/ml) (Fig 2D). Treatment with 1 and 10 ng/ml LPS had a similar but less pronounced effect (data not shown). In cultured astrocytes a comparable time dependency for the LPS effects was detected as it was found for cultured microglia (data  
10 not shown). No CCR12 mRNA expression was detected in cDNA derived from cultured cortical neurons (data not shown).

In order to verify the results obtained with RT-PCR *in situ* hybridisation was combined with immune histochemistry. Mixed glial cultures were stimulated for 2h with LPS (100 ng/ml) and stained with ED-1 and GFAP to detect microglia and astrocytes respectively.

15 ED-1 positive microglia (brown reaction product) showed a positive signal for CCR12 in situ hybridisation (purple reaction product) (Fig 3A). Note that an *in situ* signal is also visible in ED-1 negative cells, which might be in this case an astrocyte (arrowhead). (Fig. 3A). CCR12 positive astrocytes (purple reaction product) (arrows) became visible by staining with GFAP (brown reaction product) (Fig 3B). Note that also GFAP negative  
20 cells are in situ positive, which is this case most likely a microglia cell (arrowhead) (Fig 3B).

#### *Expression of CCR12 mRNA in brain tissue*

Mice were injected intraperitoneally with LPS (50ug/25g weight) or with 0.9% NaCl and  
25 brains were removed after 2, 4, 8, 12 and 24h for RT-PCR analysis or *in situ* hybridisation. Injection with control NaCl solution did not affect expression of CCR12 mRNA in brain tissue (data not shown). In contrast, injection of LPS induced the expression of CCR11 mRNA 2, 4 and 8h after the injection. 12h after the injection of LPS CCR12 mRNA expression returned to baseline levels (Fig 4). These results were  
30 verified by *in situ* hybridisation experiments. No CCR12 mRNA positive cells were found in control brains (Fig 5A), 2h after injection of LPS many CCR12 positive cells were observed in the cortex of the LPS treated mice (Fig 5B), 24h after the injection CCR12 *in situ* hybridisation signal returned to control levels (Fig 5C). Combinations of *in situ* hybridisation (purple reaction product) (Fig 5D) and immuno-histochemistry  
35 (GFAP fluorescence) (Fig 5E) revealed that GFAP positive astrocytes express CCR12

mRNA in mouse cortex (see Fig 5F for overlay of 5D and E). For technical reasons it was not possible to co-localize CCR12 mRNA with microglial markers *in vivo*. Since CCR12 mRNA positive and GFAP negative cells were found in brain is suggested that there are celltypes different from astrocytes expressing CCR12 mRNA, which might be microglia as observed in cell culture studies.

*Effect of MCP-1 and RANTES (CCL5) on chemotaxis and calcium signaling of RAW 264.7 cells*

In order to find possible chemokine ligands for CCR12 chemotactic activity and mobilisation of intracellular calcium was determined in LPS treated RAW cells. MCP-1 induced concentration-dependent chemotaxis of RAW cells with an EC50 value of approximately 0.1 nM (Fig 6A). Similar results were obtained using RANTES, which was less potent with an EC50 value of approximately 1nM (Fig 6A). The CC chemokine MIP-1 $\alpha$  (CCL3) did not induce chemotaxis in RAW cells (data not shown). Both chemokines RANTES and MCP-1 were also found to induce intracellular calcium transients in RAW cells (Fig 6B).

*Chemotaxis of CCR12 transfected HEK cells*

In order to further investigate its agonist responsivity we cloned mouse CCR12 from LPS treated microglia and subsequently the receptor was expressed in HEK 293 cells. Sequencing of the glial CCR12 revealed 99% identity with the sequence previously published for the orphan receptor (27). Mock transfected HEK cells did not migrate towards a chemotactic gradient of MCP-1, whereas CCR12 transfected HEK cells concentration dependently migrated in response to MCP-1 (Fig 7A). Moreover, MCP-1 induced intracellular calcium transients in CCR12 transfected HEK cells (Fig. 7B). Among several other chemokines found in brain (RANTES, Fractalkine (CX3CL1), MIP-1 $\alpha$ , MIP-1 $\beta$  (CCL4), MIP-3 $\alpha$  (CCL20), IP-10 (CXCL10), MCP-2 (CCL8), MCP3 (CCL7) and SLC (CCL21)) only RANTES , MCP-2 and MCP-3 were found to induce chemotaxis of CCR12 transfected HEK cells (Tab. 3). In set of preliminary experiments we performed chemotaxis assays with HEK cells expressing the human CCR12 and verified that MCP-1 is a chemokine ligand also for human CCR12. (data not shown).

*Effect of MCP-1 on calcium and chemotaxis of cultured microglia*

Stimulation of chemotaxis of cultured mouse astrocytes by MCP-1 has already been  
5 shown by Heesen et al., (1996). Effects of MCP-1 on cultured microglia were only shown  
so far for rat microglia (20, 22) and fetal human microglia (23) but not for mouse  
microglia. We therefore determined the effects of MCP-1 on intracellular calcium  
transients and chemotaxis of cultured mouse microglia. Similar to microglia from other  
species, 10 nM MCP-1 induced chemotaxis of cultured mouse microglia; migration of  
10 untreated cells,  $29 \pm 13$  (cells/mm<sup>2</sup>), migration of cells stimulated with 10 nM MCP-1  
 $170 \pm 42$  (cells/mm<sup>2</sup>) (n=4). Chemotaxis was determined as described in materials and  
methods. Moreover intracellular calcium transients in cultured microglia were observed  
upon stimulation with MCP-1 (data not shown).

Table 1

Primer sequences for mouse CCR's

Gene	Primer sequences (5'-3')	PCR product (bp)
CCR1	GTGGTGGGCAATGTCCTAGT TCAGATTGTAGGGGGTCCAG	658
CCR2	GTATCCAAGAGCTTGATGAAGG G  GTGTAATGGTGATCATCTTGTTT GGA	532
CCR3	GCACCACCCTGTGAAAAAGT CGAGGACTGCAGGAAAACTC	521
CCR4	AGGCAAGGACCCTGACCTAT GGACTGCGTGTAAGAGGAGC	644
CCR5	ATTCTCCACACCCTGTTTCG TCAGGCTTGTCTTGCTGGAA	350
CCR6	GTGGTGATGACCTTTGCCTT AGGAGGACCATGTTGTGAGG	656
CCR7	AACGGGCTGGTGATACTGAC ATGAAGACTACCACCACGGC	596
CCR8	TTCCTGCCTCGATGGATTAC GCTTCCACCTCAAAGACTGC	591
D6	TCTTCATCACCTGCATGAGC TATGGGAACCACAGCATGAA	400
CCR12	CTGGCGGTGTTTATCTTGGT AACCAGCAGAGGAAAAGCAA	489
GAPDH	CATCCTGCACCACCAACTGCTTA G GCCTGCTTCACCACCTTCTTGAT	346

	G	
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Table 2

Comparison of CCR12 with all other cloned mouse CCR's by nucleic acid sequence alignment

5

Beta chemokine receptor (mouse)	Accession Number	Percentage ID after alignment with glial CCR12
CCR1	U28404	52.8
CCR2	U51717	49.6
CCR3	U29677	54.3
CCR4	X90862	51.5
CCR5	U83327	53.1
CCR6	AB009369	51.6
CCR7	L31580	50.2
CCR8	Z98206	56.6
CCR9	AJ132336	49.1
CCR10	AF215982/ AF215983	48.0
D6	Y12879	50.0



Table 3

Effect of various chemokines on chemotaxis of CCR12 transfected HEK 293 cells

Chemokine (100nM)	Chemotactic effect on CCR12 transfected HEK cells
RANTES	+
MCP-1	+
MCP-2	+
MCP-3	+
MIP-1 $\alpha$	-
MIP-1 $\beta$	-
MIP-3 $\alpha$	-
Fractalkine	-
IP-10	-
SLC	-

Table 4

Expression profile of CCR mRNA in cultured glial cells from human (H), mouse (M) and rat (R).

5

	astrocytes			microglia			publication
	H	M	R	H	M	R	
CCR1		+				+	19
				+			20
	-			+			32
CCR2		-				+	24
						-	20
						-	18
	-		-	+			32
CCR3			-			-	18
						-	20
				+			33
	-			+			32
CCR4		-				-	19
						-	20
	-			-			32
CCR5			-			+	18
						+	20
				+			33
	-			+			32

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## Legends to the figures

### Figure 1

RT-PCR analysis of CCR12 mRNA expression in unstimulated (C) and LPS stimulated  
 5 RAW 264.7 cells. Cells were stimulated with 100ng/ml LPS for 2h. Number of cycles for  
 GAPDH and CCR12 were 28 and 32 respectively. MM, molecular weight marker,  
 highlighted band is 500bp. Both PCR products were run in the same gel. Similar results  
 were found in 3 independent experiments.

### Figure 2

RT-PCR analysis of chemokine receptor mRNA expression in cultured microglia (A and  
 B) and cultured astrocytes (C and D). Experiments were carried out as described in  
 materials and methods. A) CCR1, 3 and 5 mRNA was found in cultured microglia. B)  
 Unstimulated microglia (C) did show basal CCR12 mRNA expression which was  
 15 upregulated by a 2h stimulation with 100ng/ml LPS. C) In cultured astrocytes mRNA  
 expression for CCR1 and 5 was found. D) Control astrocytes (C) did show basal  
 expression of CCR12 mRNA which was upregulated by a 2h stimulation with 100ng/ml  
 LPS. Number of cycles for GAPDH and CCR12 were 28 and 32 respectively. MM,  
 molecular weight marker, highlighted band is 500bp. For B and D were both PCR  
 20 products run in the same gel. Similar results were found in 3 independent experiments.

### Figure 3

In situ hybridisation in combination with immuno histochemistry shows CCR12 mRNA  
 expression in LPS stimulated cultured microglia and astrocytes. Cultured glial cells  
 25 were stimulated for 2h with LPS (100ng/ml) and fixed as described in material and  
 methods. A) Cells were incubated with ED-1 antibody to stain microglia (brown reaction  
 product). The combination with in situ hybridisation (purple reaction product) revealed  
 that ED-1 positive microglia also express CCR12 mRNA (arrows). The ED-1 negative but  
 CCR12 mRNA positive cell might be an astrocyte (arrowhead). B) Cells were incubated  
 30 with GFAP antibody to stain astrocytes (brown reaction product). The combination with  
 in situ hybridisation (purple reaction product) clearly showed that GFAP positive cells  
 also express CCR12 mRNA (arrows). The GFAP negative but CCR12 mRNA positive cell  
 is most likely a microglia (arrowhead). Bar 10um.

#### Figure 4

Effect of LPS injection on CCR12 mRNA expression in mouse brain. RT-PCR experiments revealed that CCR12 mRNA expression in mouse brain was induced 2, 4 and 8h after the injection of LPS (50ug/25g weight). 12h after the injection CCR12 mRNA expression returned to control levels. Number of cycles for GAPDH and CCR12 were 28 and 32 respectively. MM, molecular weight marker, highlighted band is 500bp. Both PCR products were run in the same gel. Similar results were found in 3 independent experiments.

#### Figure 5

CCR12 mRNA in situ hybridisation in the cortex of LPS injected mice and identification of astrocytes as CCR12 mRNA expressing cells. A) Lack of CCR12 mRNA expression in control brain, only unspecific staining is visible. B) 2h after the injection of LPS CCR12 mRNA expression is induced in many cells. C) CCR12 mRNA returned to control levels 24h after the injection of LPS. D) CCR12 mRNA positive cells in higher magnification in mouse brain 2h after LPS injection. E) Fluorescence micrograph of the same region as in D stained with anti-GFAP to detect astrocytes. F) Electronic overlay of D and E to verify that some CCR12 positive cells stain for GFAP indicating that astrocytes are a cellular source of CCR12 mRNA. Note that there are also CCR12 mRNA positive and GFAP negative cells indicating that at least one other cell type different from astrocytes express CCR12 mRNA. Bar in A-C 50um; in D-F 10um.

#### Figure 6

Effects of MCP-1 and RANTES on chemotaxis and intracellular calcium transients of cultured RAW 264.7 cells. A) Concentration-dependent chemotaxis of cultured RAW cells induced by MCP-1 and RANTES. The graphs show the results of a typical chemotaxis experiment performed in hexaplicate for each concentration of MCP-1 and RANTES. Data are means  $\pm$  SEM (n=4); similar results were obtained in 4 independent experiments. B) Figure shows a typical example of an induction of intracellular calcium transients in RAW cells by MCP-1 or RANTES, arrow indicates the timepoint of stimulation.

#### Figure 7

Effect of MCP-1 on chemotaxis and intracellular calcium transients of CCR12 transfected HEK cells. A) Chemotaxis of MOK-transfected HEK cells was not affected

by MCP-1, whereas CCR12 transfected HEK cells migrated concentration dependend when stimulated with MCP-1. The graphs show the results of a typical chemotaxis experiment performed in hexaplicate for each concentration of MCP-1. Data are means  $\pm$  SEM; similar results were obtained in 3 independent experiments. B) Typical example  
5 of a MCP-1 (100nM) induced intracellular calcium transient in CCR12 transfected HEK cells.

#### Figure 8

- 10 Multiple nucleotide sequence alignment of human (hCCR12) or mouse (mcCCR12) chemokine receptor sequences herein addressed as CCR12



18.01.2001

## Claims

(100)

1. A method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising testing said compound for its capacity to modulate or mimic MCP-1 binding with a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CCR2-B.
2. A method according to claim 1 wherein said disease comprises ischemia, Alzheimer's disease or multiple sclerosis.
3. A method according to claim 1 or 2 wherein said capacity to modulate or mimic MCP-1 binding further comprises down-regulation of said receptor.
4. A method according to claim 3 wherein said capacity is tested *in vitro*.
5. A method according to claim 4 wherein mRNA expression of said receptor is up-regulated.
6. A method according to claim 5 wherein said expression is upregulated by treatment with lipopolysaccharide (LPS).
7. A method according to anyone of claims 1 to 6 wherein said capacity to modulate or mimic MCP-1 binding is measured by determining chemotaxis.
8. Use of a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CCR2-B, or functional equivalent thereof in a method according to any one of claims 1 to 7.
9. Use according to claim 8 wherein said receptor or functional equivalent thereof is expressed in a cultured cell.
10. Use according to claim 9 wherein said cultured cell comprises a cell transfected with a nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CCR2-B, or functional equivalent thereof.

11. Use according to claim 10 wherein said cell comprises a HEK cell.

12. A cell comprising a recombinant nucleic acid encoding at least a functional  
5 fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or  
functional equivalent thereof.

13. An animal comprising a cell according to claim 12.

10 14. A method for obtaining or identifying an agonist or antagonist of  
neurodegenerative of neuroinflammatory disease comprising testing a candidate agonist  
or antagonist compound in a method according to any one of claims 1 to 7 and  
determining said compound's capacity to modulate or mimic MCP-1 binding to said  
receptor in said method.

15 15. An agonist or antagonist of neurodegenerative of neuroinflammatory disease  
obtainable or identifiable by a method according to claim 14.

16. Use of an agonist or antagonist according to claim 15 for the preparation of a  
20 pharmaceutical composition.

17. Use of claim 14 for the preparation of a pharmaceutical composition for the  
treatment of neurodegenerative of neuroinflammatory disease.

25 18. A pharmaceutical composition comprising an agonist or antagonist according to  
claim 15.

19. A method for the treatment of a neurodegenerative of neuroinflammatory disease  
comprising treating an individual with a pharmaceutical composition according to claim  
30 18.

## Abstract

18. 01. 2001



The invention relates to the fields of inflammation and immunology, and more  
5 specifically to the field of chemokines and receptors therefor, and their role in  
neurodegenerative or neuroinflammatory disease. The invention provides a method for  
identifying a candidate drug compound for the treatment of inflammatory or  
degenerative brain disease comprising testing said compound for its capacity to  
modulate or mimic MCP-1 binding with a chemokine receptor capable of being  
10 expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans  
as CCR4-B.



18. 01. 2001

100

FIGURE 1

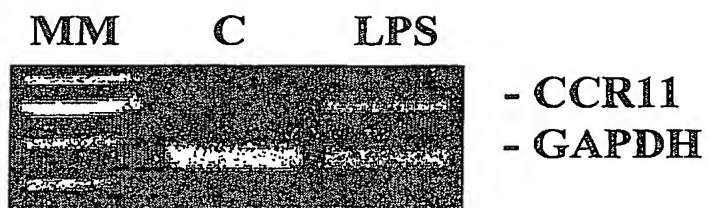


FIGURE 2

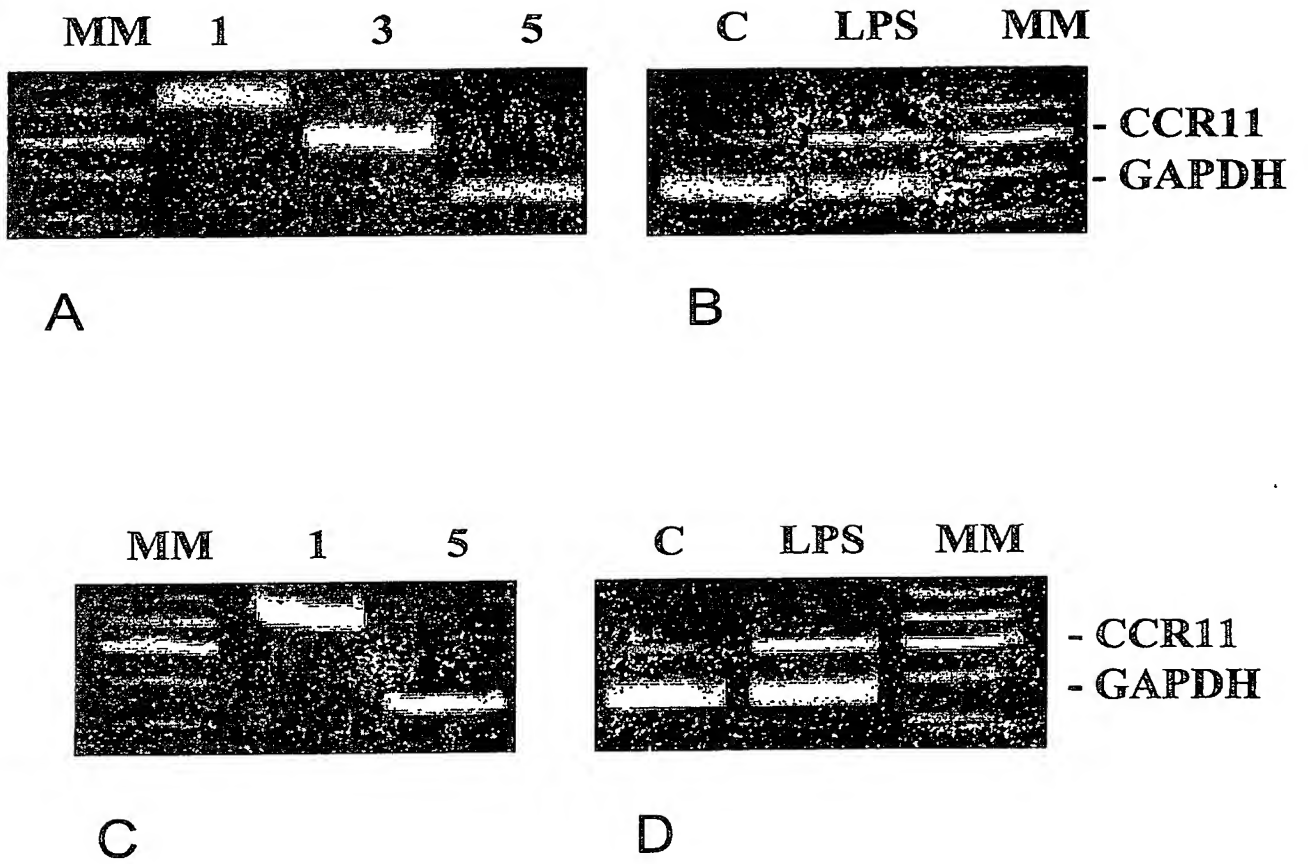


FIGURE 3

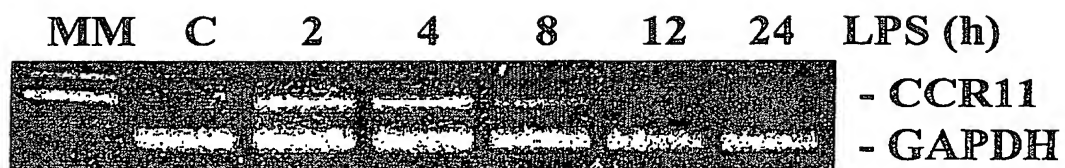


FIGURE 4

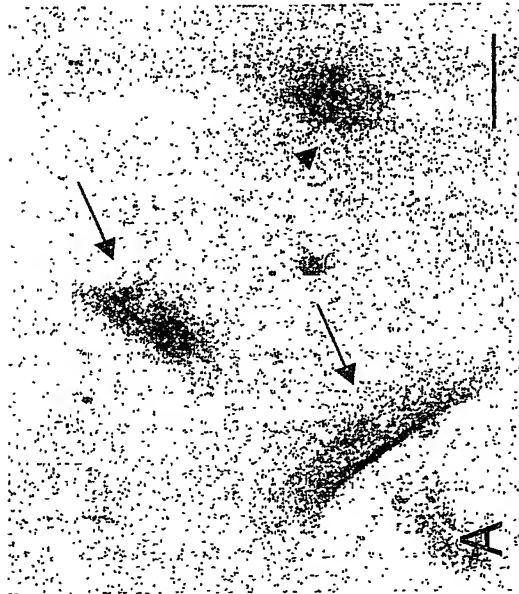




FIGURE 5

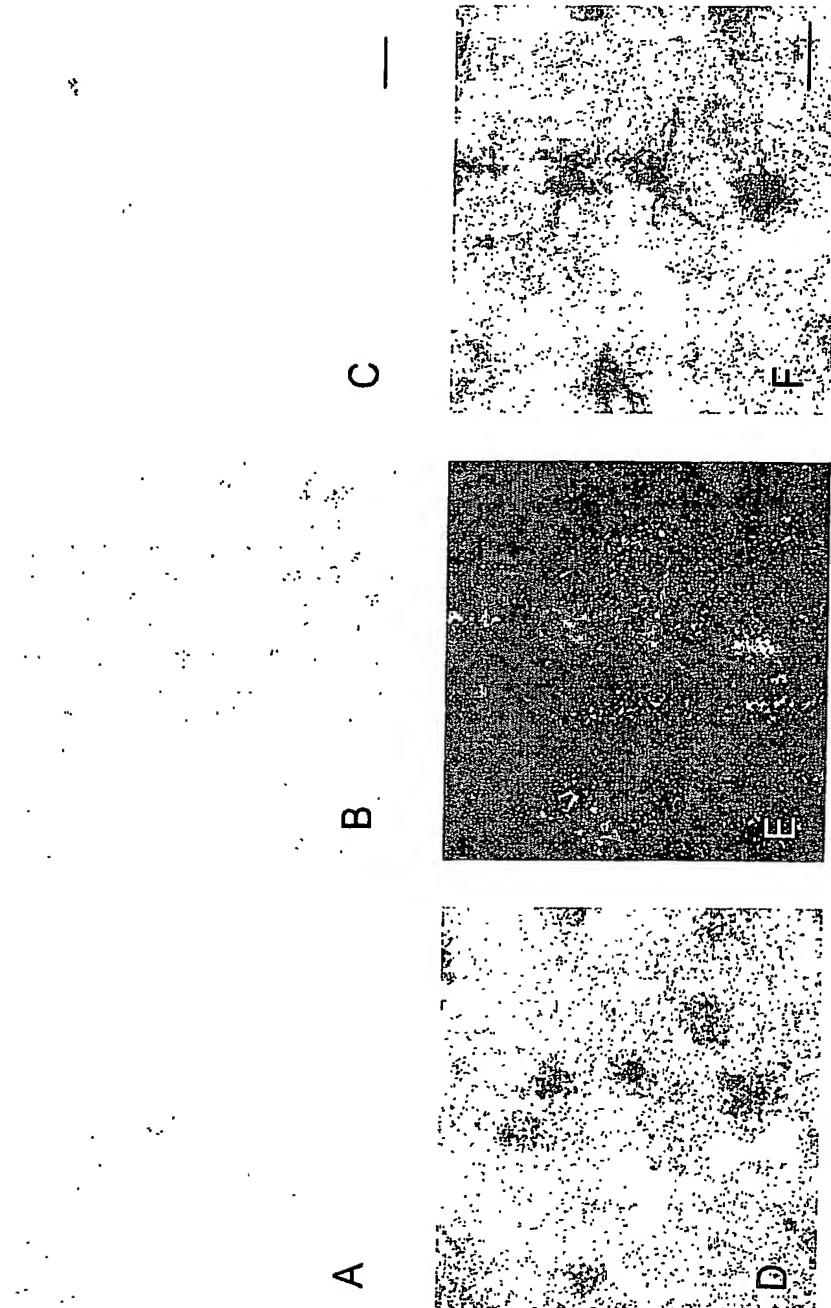
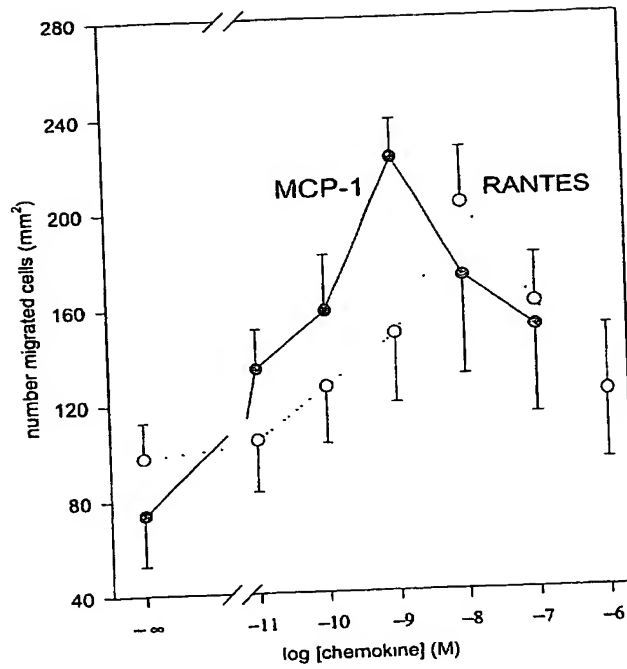
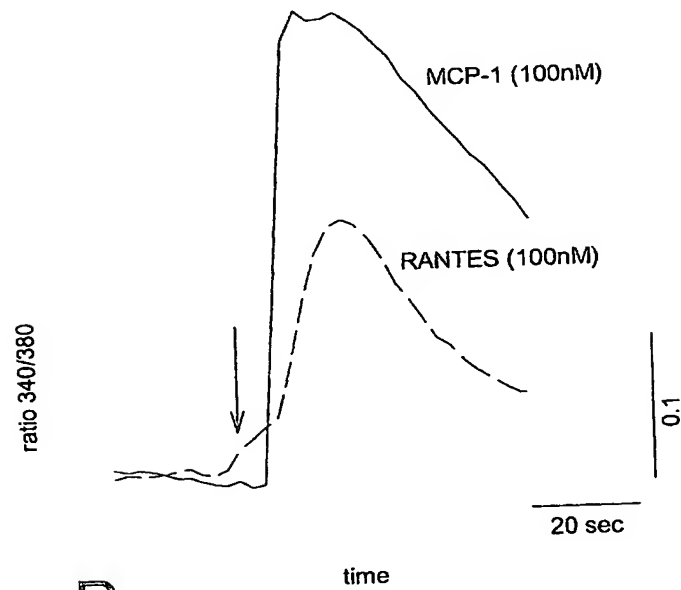


FIGURE 6

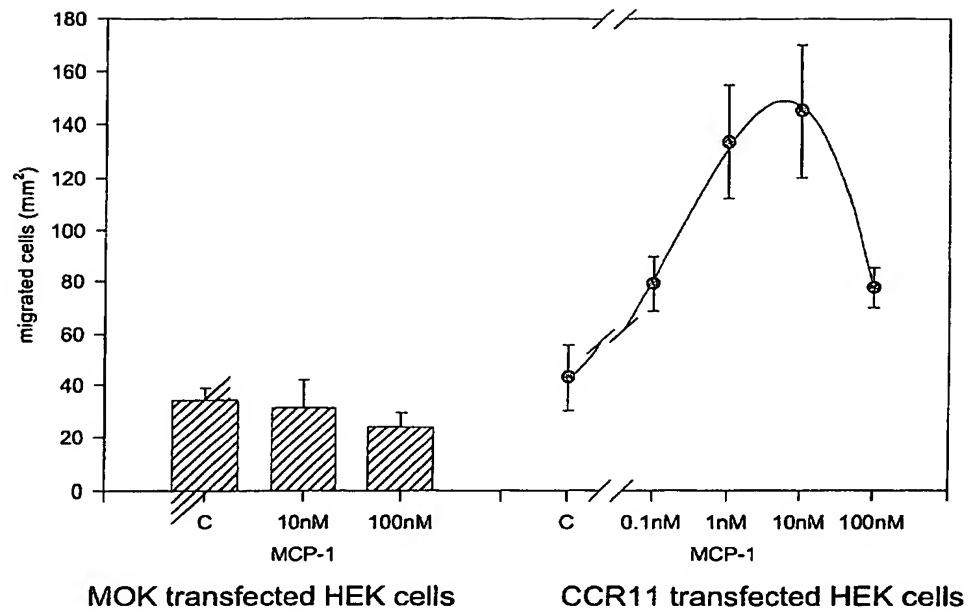


A

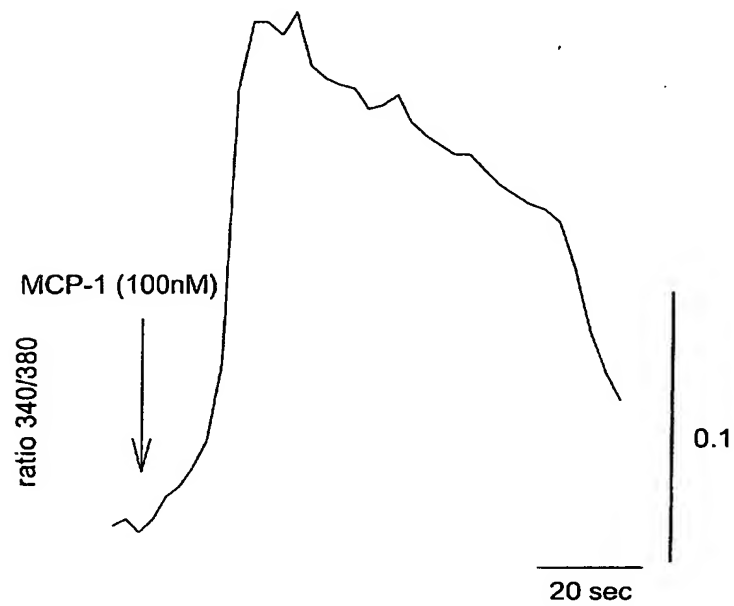


B

FIGURE 7



A



B

FIGURE 8

CLUSTAL W (1.81) multiple sequence alignment

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hCCR12      ATGCCCAATTACACGCTGGCACCAGAGGATGAATATGATGTCCTCAT---AGAAGGTGAA 57
mCCR12      ATGGATAACTACACAGTGGCCCGGACGATGAATATGATGTCCTAATCTTAGACGACTAC 60
          *** ** ***** ** ** ***** ** ** *
          *** ** ***** ** ** ***** ** ** *

hCCR12      CTGGAGAGCGATGAGGCAGAGCAATGTGACAAGTATGAGCCCGAGGCACCTCTCAGCCAG 117
mCCR12      CTGGACAACAGTGGGCCGACCAA-GTTCCG-----GCCCGGAGTTCCTCTCCCCCAG 114
          ***** * * * * * * * * * * * * * * *
          ***** * * * * * * * * * * * * * * *

hCCR12      CTGGTGCCATCACTCTGCTCTGCTGTGTTTGTGATCGGTGTCGTGGACAATCTCCTGGTT 177
mCCR12      CAGGTGCTGCAGTTCTGCTGCGCGGTGTTTGTGCGGTGGGTCTCTTGGACAACAGTGTGGCG 174
          * ***** ** ***** * * * * * * * * *
          ***** ** ***** * * * * * * * * *

hCCR12      GTGCTTATCCTGGTAAATATAAAGGACTCAAACGCGTGGAAAATATCTATCTTCTAAAC 237
mCCR12      GTGTTTATCTTGGTGAAATACAAAGGACTCAAGAATCTGGGGAACATCTACTTCTCTAAAC 234
          *** ***** ** ***** ** ** ***** * *****
          ***** ** ***** ** ***** ** *****

hCCR12      TTGGCAGTTTCTAACTTGTTGTTTCTTGTCTTACCCCTGCCCTTCTGGGCTCATGCTG---- 292
mCCR12      CTGGCACTTTCAAAACCTGTGTTTCTGTTCCCTTCCCTGCCGTCTGGGCCCATACTGCAGCA 294
          ***** ** ***** ** ***** ** ***** ** *****
          ***** ** ***** ** ***** ** *****

hCCR12      ---GGGGCGATCCC-----ATGTGTAATAATTCTCATTTGGAAGTCTTCTCGTGGGC 339
mCCR12      CACGGGGAAAGCCCTGGCAACGGGACCTGTAAAGTTCTTGTCCGACTCCACTCCTCGGGC 354
          **** * *** * ***** ** * ***** *** * *****
          **** * *** * ***** ** * ***** *** * *****

hCCR12      CTGTACAGTGAGACATTTTCAATTGCCCTTCTGACTGTGCAAGGTACCTAGTGTTTTG 399
mCCR12      TTATACAGCGAGGTGTTTCCCAACATCCTCCTCTGTCGCAAGGATACAGGGTGT--- 412
          * ***** *** ***** *** ** ***** * * * * *
          * ***** *** ***** *** ** ***** * * * * *

hCCR12      CACAAGGGCAACTTTTCTCAGCCAGGAGGAGGTGCCCTGTGGCATCATACAAGTGTC 459
mCCR12      CCCAAGGGCGACTGGC-CTCCATCTTTCACGACAGTGTCTTGTGGTATTGTGCGTGCATC 471
          * ***** *** ** * * * * * * * * * * * * * * *
          * ***** *** ** * * * * * * * * * * * * * * *

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hCCR12	CTGGCATGGGTAAACAGCCATTCTTGCCCACTTTTGCCTGAATTCGTGTTTTATAAACCTCAG	519
mCCR12	CTGGCATGGGCCATGGCTACTGCGCTCTCTTGGCCCCGAGTCTGTGTTTTATAGACCCTCGG	531
	***** * ** * * * ***** ** * *** ***** * **** *	
hCCR12	ATGGAAGACCAGAAAATACAAGTGTGCATTTAGCAGAACCTCCCTTCTTGCCAGCTGATGAG	579
mCCR12	ATGGAAGACAGAAAAACACAAGTGTGCCCTTTGGCAAACCTCACITTTTGCCAATCGAAGCG	591
	***** ***** ***** ** * *** * *** ***** ** * *	
hCCR12	ACATTCTGGAAGCAATTTCTGACTTTTAAATAAGAACATTTCCGTTCTTGTCTCCCCCCTA	639
mCCR12	CCGCTCTGGAAGTAGCTTCTGACGTC AAAAATGATCATCTTGGTACTTGCTTTTCTCTCTG	651
	* ***** * ***** * ***** ** * *** * *** * ** *	
hCCR12	TTTATTTTACATTTCTCTATGTGCAATAGAAAAACACTAAGGTTTCAGGGAGCAGAGG	699
mCCR12	CTGGTTTTTATAATCTGCTGCAGGCAACTGAGGAAGGCAGAGCTTCAGGGAGAGACAG	711
	* ***** * * ** ***** ***** * ** ******	*
hCCR12	TATAGCCTTTTCAAGCTGTTTTTGCCGTAATGGTAGTCTTCCCTTCGATGTGGGCGCCC	759
mCCR12	TACGACCTCCACAAGCGCGCTCTTGTGCATAACGGGCGTGTTCCTTTTGATGTGGGCGCCT	771
	** ***** * * *** * * *** ** * ***** ******	
hCCR12	TACAAATATTGCATTTTCTGTCCACTTTC AAGAAACACTTCTCCCTGAGTGACTGCAAG	819
mCCR12	TACAACACTGTGCTTTTCTGTCTGCTTTC CAGGAACACTTGTCCCTGAGGATGAGAAG	831
	***** * ** ***** ***** ***** * ***** ** *****	***
hCCR12	AGCAGCTACAATCTGGACAAAAGTGTTCACATCACTAAACTCATCGCCACCACCCACTGC	879
mCCR12	AGCAGCTACCACCTGGACGCAAGTGTTCAGGTACACAGCTGGTAGCGACCACCCACTGC	891
	***** * ***** ***** ***** * ** * ** ******	
hCCR12	TGCATCAACCTCTCTGTATGCGTTTCTTGATGGGA-- -CATTTAGCAATAACCTCTGC	936
mCCR12	TGCGTCAACCCGCTGCTATTGTCTTCTTGACGGGAAGGCCCTTTATGAGATACCTTCGC	951
	*** ***** ** ** *** * ***** ** * ***** * ***** *	***

FIGURE 8, Contd.

hCCR12	CGCTGTTTCCATCTGTGCGTAGTAACACCCCACTTCAACCCAGGGGGCAGTCTGCACAAGGC	996
mCCR12	AGCCTGTTCCCAAGGTGCAATGATATCCCTATCAAAAGTAGTGGAGGCTATCAGCAAGCG	1011
	** **** * * * * * **** * * * * *	
hCCR12	ACATCGAGGGGAAGAACCTGACCATTTCCACCGAAGTGTA	1035
mCCR12	CCTCCAAGGGGAAGGTCAATGGCAGGCCCATTTGAACGTGTACAGCAATTTGCATCAAGGCAG	1071
	* * ***** * * * * * * * * * *	
hCCR12	-----	
mCCR12	GATATAATATAA	1083